

Abnormal muscle and hematopoietic gene expression may be important for clinical morbidity in primary hyperparathyroidism

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Submitted 11 September 2006; accepted in final form 12 January 2007

Reppe S, Stilgren L, Abrahamsen B, Olstad OK, Cero F, Brixen K, Nissen-Meyer LS, Gautvik KM. Abnormal muscle and hematopoietic gene expression may be important for clinical morbidity in primary hyperparathyroidism. *Am J Physiol Endocrinol Metab* 292: E1465–E1473, 2007. First published January 16, 2007; doi:10.1152/ajpendo.00487.2006.—In primary hyperparathyroidism (PHPT), excess PTH secretion by adenomatous or hyperplastic parathyroid glands leads to elevated serum $[Ca^{2+}]$. Patients present complex symptoms of muscular fatigue, various neuropsychiatric, neuromuscular, and cardiovascular manifestations, and, in advanced disease, kidney stones and metabolic bone disease. Our objective was to characterize changes in muscle and hematopoietic gene expression in patients with reversible mild PHPT after parathyroidectomy and possibly link molecular pathology to symptoms. Global mRNA profiling using Affymetrix gene chips was carried out in biopsies obtained before and 1 yr after parathyroidectomy in seven patients discovered by routine blood $[Ca^{2+}]$ screening. The tissue distribution of PTH receptor (PTHR1 and PTHR2) mRNAs were quantitated using real-time RT-PCR in unrelated persons to define PTH target tissues. Of about 10,000 expressed genes, 175 muscle, 169 hematological, and 99 bone-associated mRNAs were affected. Notably, the major part of muscle-related mRNAs was increased whereas hematological mRNAs were predominantly decreased during disease. Functional and molecular network analysis demonstrated major alterations of several tissue characteristic groups of mRNAs as well as those belonging to common cell signaling and major metabolic pathways. PTHR1 and PTHR2 mRNAs were more abundantly expressed in muscle and brain than in hematopoietic cells. We suggest that sustained stimulation of PTH receptors present in brain, muscle, and hematopoietic cells have to be considered as one independent, important cause of molecular disease in PHPT leading to profound alterations in gene expression that may help explain symptoms like muscle fatigue, cardiovascular pathology, and precipitation of psychiatric illness.

microarray; biopsies; parathyroid hormone

THE PREVALENCE OF PRIMARY HYPERPARATHYROIDISM (PHPT) varies by age and sex, affecting 1 in 500 females and 1 in 2,000 males over the age of 40 yr (7), or ~1% of the adult population (19). The prevalence of PHPT increases with age in both males and females. PHPT is usually due to benign tumors or hyperplasia affecting one or several parathyroid glands, which therefore secrete constitutively too much parathyroid hormone (PTH), leading to increased levels of blood calcium. Loss of calcium from the skeleton leads to osteoporosis (OP) and

osteomalacia (26). There is a growing recognition that symptoms rising early from organ systems other than bone and kidneys are important and add to the morbidity of patients with PHPT in addition to the more classical findings of nephrolithiasis and osteopenia (48). Many reports (14, 23, 24, 42, 43) emphasize the change in the clinical presenting picture from renal, bone-related, and gastrointestinal manifestations to psychological and psychiatric disorders as well as feelings of muscular weakness, apathy, and ill-defined mental symptoms. An increased cardiovascular morbidity has been reported even in mild disease (2), leading to overrepresentation of cardiac death in patients with symptomatic PHPT both before and after parathyroidectomy (PTX), suggesting development of nonreversible changes. The actions of PTH are mediated through three types of receptors: PTH/PTH-related peptide receptor (PTHR1), the NH₂-terminal PTH receptor II (PTHR2), and the COOH-terminal PTH receptor (C-PTHR) (22, 38, 46, 47). In humans, PTHR1 mRNA is reported to be widely expressed in tissues like brain, striated muscle, heart muscle, kidney, and liver (38, 46). The PTHR2 mRNA shows a more restricted distribution and has lower affinity for PTH compared with PTHR1, but it is present in blood cells (39) and abundant in the brain and pancreas (47). The COOH-terminal part of PTH is essential for binding to the C-PTHR receptor, which has not yet been cloned but is highly expressed, e.g., in rat osteocytes (10).

Very little is known about the molecular pathology associated with a chronic excessive action exerted by PTH in different target tissues in patients with PHPT. The aim of the present study was to describe in detail changes in gene expression in biopsies from patients with well-defined and characterized early PHPT before and after surgery when normalization of bone and biochemical markers had occurred. We used each patient as his/her own control to describe the molecular pathology expressed as mRNA profiles and suggest that the results may have clinical consequences reflecting chronic PTH receptor stimulation.

METHODS

Patients and biopsies. Seven arbitrarily chosen patients from 53 to 75 yr of age (mean 60.3 yr), without signs of organ affection but with clinical biochemical evidence of mild PHPT, were included. Biochemical blood diagnostic and bone parameters for the patients have been described previously (35). Briefly, PTH, ionized calcium, and 1,25(OH)₂D, as well as bone formation and resorption markers, and

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were all significantly reduced 1 yr after surgery, whereas bone mineral density levels in spine and hip increased significantly. The diagnosis was established by elevated plasma PTH and ionized calcium, taking care to rule out accessory conditions known to affect the PTH/calcium balance. The patients were screened for interfering conditions such as cardiovascular and renal disorders, endocrine disorders, and other bone pathology. Vitamin D status [25(OH)D and 1,25(OH)2D] was evaluated before inclusion. The patients had normal serum creatinine levels. Following inclusion in this study, the patients underwent successful PTX.

Biopsies containing bone, bone marrow, and remnants of skeletal muscle tissue were taken from opposite but symmetrical places of os ileum to avoid woven bone from the first biopsy before and 1 yr after surgery. Biopsies were immediately frozen in liquid nitrogen and stored at -70°C for RNA extraction.

The distribution of PTHR1 and PTHR2 in normal tissues and blood cells, was studied in biopsies/blood samples obtained from other patients taken during surgery. The receptors in central nervous system (CNS) were analyzed in post mortem tissue from nondemented humans (obtained from Netherlands Brain Bank, Amsterdam, The Netherlands). The study was performed according to Title 45, US Code of Federal Regulations, Part 46, "Protection of Human Subjects," and to the declaration of Helsinki II and approved by the Local Ethics Committee (Ethics ref no. 19980012). Informed written consent was obtained from each PHPT participant and other healthy persons before entry. Also the examination of post mortem specimens fulfilled all formal requirements.

Purification of RNA. RNA from biopsies and autopsies was purified with the aid of TRIzol (Life Technologies, Gaithersburg, MD) and RNeasy (Qiagen), as described (35). Isolation of lymphocytes and monocytes was performed as described (31), and RNA from the purified cell fractions was isolated with the aid of TRIzol. The RNA quality was controlled by gel electrophoreses.

Microarray analysis. Double-stranded cDNA and biotin-labeled cRNA probes were made from 5 μg of total RNA by use of the Superscript Choice system (Invitrogen) and the Enzo Bioarray, respectively, according to recommendations from Affymetrix. This cRNA was hybridized to HG-133A chips (Affymetrix) followed by washing and staining on the GeneChips Fluidics Station 450 (Affymetrix). The chips were scanned on the Affymetrix GeneArray 2500 scanner. The quality of the RNA and probe was controlled by an Affymetrix-based test measuring the ratio between 5' and 3' mRNAs for β -actin and GAPDH and found to be highly satisfactory. The datasets originating from the 14 bone biopsies were processed by the Affymetrix Mas 5.0 software according to manufacturer's instructions.

The patients were coded, and all analyses were carried out blindly. The samples from each patient were analyzed using the same kit for cRNA probe synthesis and hybridized to chips from the same batch. Intrabatch chip variation was found to be negligible. One patient, *patient 7*, showed different overall mRNA profiles for genes related to muscle, hematological, and bone matrix and was analyzed on two chips from different batches to rule out technical causes. In general, the differences in expression levels before/after operation were less for *patient 7*. Of the 175 mRNAs related to muscle, 123 were on average more than twofold changed in *patients 1–6* compared with only 24 in *patient 7*. Of the 169 mRNAs related to hematopoiesis, 46 were on average more than twofold changed in *patients 1–6* compared with only 15 in *patient 7*. Furthermore, 96 and 80% of muscle- and hematopoiesis-related mRNAs increased or decreased, respectively, on average in *patients 1–6*, whereas the corresponding numbers in *patient 7* were 14 and 44%, respectively.

Filtering and statistical analysis of Affymetrix data. First, the genes were sorted by criteria based on uniformity of expression among *patients 1–6*. Second, the average acceptable signal level before or after PTX was set to be above 50 to include the probe set. Third, for inclusion in the statistical analysis, changes reflecting a mean increase

or decrease of at least 40% (representing a log2 value of more than 0.5 or less than -0.5) were required. *Patient 7* was treated as a special case because she was considered to represent a different entity (see RESULTS). Two statistical criteria were employed. For *criterion 1*, the ratio of mRNA values between "sick" and "cured" for each probe set (transcript) was given a *P* value by the Affymetrix Mas 5.0 program. The median *P* value for one transcript from six patients was required to be 0.05 or better according to the 0-hypothesis. For *criterion 2*, the ratio of mRNA values between "sick" and "cured" were given a *P* value by the Affymetrix Mas 5.0 program for each gene, and values were then used in the empirical Bayes method for evaluation of the significance of regulation (33, 41, 49).

Data evaluation using the Ingenuity Pathways Analysis program. The canonical pathways that were identified using the Ingenuity Pathways Analysis (IPA) program were evaluated employing the right-tailed Fisher's exact test to calculate levels of significance. The *P* value for each pathway was calculated by comparing the number of user-specified genes of interest (i.e., the regulated genes) that participated in a given function or pathway relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the Ingenuity Pathways knowledge base. Only annotations that have more functions/canonical pathways analysis genes than expected by chance ("right-tailed" annotations) were used. Although the number of genes associated with a given function/pathway is an important measure when calculating the *P* value in global analyses, the *P* value is not simply proportional to this number but takes into account how much information for these genes can be found in the Ingenuity functional/pathway annotations. The probe sets of the Affymetrix chips that were used covered more than 80% of the genes in 106 of 136 metabolic pathways covered by Ingenuity.

Real-time RT-PCR analysis. cDNA was synthesized from the same RNA as was used for Affymetrix analysis using the High Capacity cDNA Archive Kit (Applied Biosystems, Stockholm, Sweden) according to the manufacturer's specifications. Real-time RT-PCR was performed using the ABI PRISM 7900HT Sequence Detection System and the 7900HT Micro Fluidic Card containing eight ports (PE Applied Biosystems), using probes labeled with the fluorescent dye FAM. TATA-binding protein (TBP) mRNA was included in the reactions and used as internal standard since the variation between signal values in each patient pre- and postsurgery was found to be small [average 2.8% (SD 16.4%)]. Predesigned primers and a probe labeled with the reporter fluorescent dye VIC, specific for TBP, were used. The cDNA was amplified under the following conditions: 50°C for 2 min, 94.5°C for 10 min, followed by cycles at 97°C for 30 s and 59.7°C for 1 min. The relative amount of mRNA for each gene was calculated using the comparative C_T method "Separate Tubes" according to manufacturer's instructions and adjusted and calculated relative to the expression of TBP mRNA.

The genes and assay IDs selected for analysis are included in Fig. 4, A and B. The distributions of PTHR1 and PTHR2 were analyzed by real-time RT-PCR employing the LightCycler and the Fast Start Master SYBR Green kit (cat. no. 2239264, Roche Diagnostics) according to the manufacturer's instructions. One sample from each organ/region from three different persons was analyzed in triplicate. Cycling profile was 94°C for 5 min, then 40 cycles of 60°C for 30 s, 72°C for 30 s, and 95°C for 30 s, and 3 min at 72°C . Gene expression was normalized to β -actin. Primers were as follows: β -actin, forward GCTACAGCTTACCACCACA, reverse GCCATCTCTTGCTC-GAAGTC; PTHR1, forward GTCCCTGAGACCTCGGTGTA, reverse AGTACCGGAAGGTGCTCAA; PTHR2, forward ATAGT-GGGAGGCAGGGAGAT, reverse TTGGCATCCTTCAGTGTCTG.

RESULTS

Individual mRNA expression profiles in patients with PHPT before and after PTX. The mRNA levels were analyzed in biopsies from seven patients by use of the Affymetrix HG-

U133A array containing more than 22,000 probe sets for 14,500 different genes. Approximately 10,000 genes were found to be expressed in all the samples according to the threshold of acceptance described in METHODS. The mRNAs were categorized as tissue characteristic, but not necessarily tissue/cell specific, by annotations from the NetAffx analysis center. Figure 1A summarizes the overall number of muscle (175) and hematopoietic tissue (169)-associated mRNAs, leaving a large group of tissue-uncharacterized mRNAs or unknown species. A total of 99 mRNAs were characteristic of bone and extracellular matrix, as described previously (35).

Employing both *criteria 1* and *2* for filtering and statistical analysis (METHODS), 139 individual muscle- and 88 hematopoiesis-related mRNAs were detected. For *patients 1–6*, a highly corresponding profile was found, in which the majority of mRNAs were increased in muscle (Fig. 1B, *a*) and decreased in hematopoietic tissue (Fig. 1B, *b*). Many of the muscle and hematopoietic transcripts were more than fourfold increased and 50% decreased, respectively, due to PHPT (Fig. 1B, *a* and *b*).

Patient 7, however, showed a different mRNA expression pattern of both hematopoiesis- and muscle-related genes compared with *patients 1–6* (see METHODS for detailed assessment) and was therefore regarded as an outlier. The different data set for *patient 7* was not due to chip variations, as they are all from the same batch as are also the kits used in the other processes, nor was it due to differences in mRNA quality. *Patient 7* probably represents a biological variant or possibly a different disease stage; she was also much older than the other patients (75 yr old compared with a mean age of 57.9 yr for the remaining 6 patients).

Expression levels of PTHR1 and PTHR2 mRNAs in bone biopsies from patients with PHPT and their distribution in human tissues of unrelated persons. Real-time RT-PCR of PTHR1 mRNA showed a 2.0 ± 0.9 -fold increase in PHPT, whereas PTHR2 mRNA expression was unaltered, confirming the Affymetrix data (35). The discovery of differential PTHR1 mRNA expression and the knowledge that PHPT precipitates symptoms from various organs prompted us to study receptor mRNA distribution in CNS, muscle, and blood cells. PTHR1

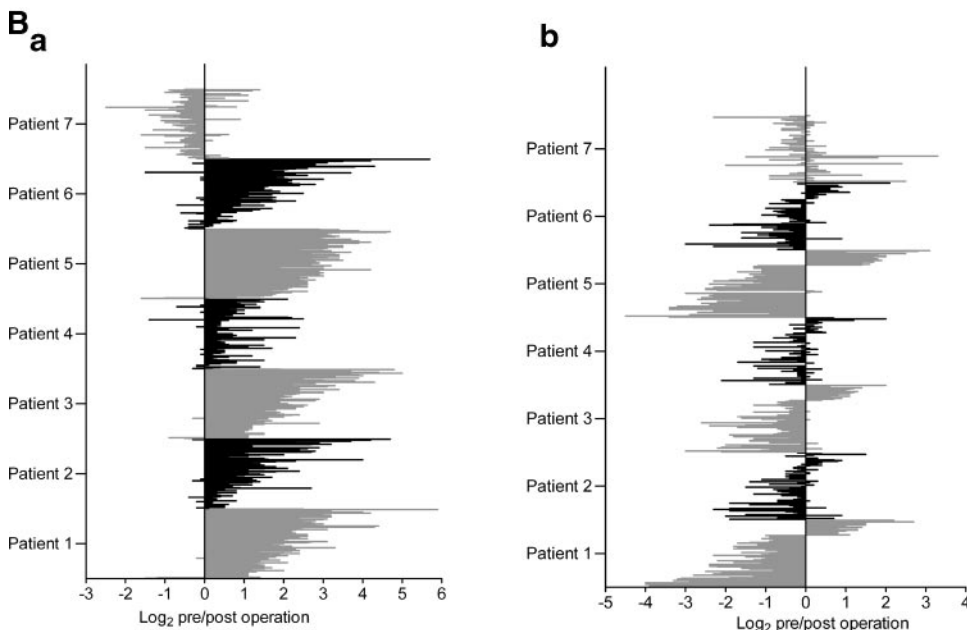
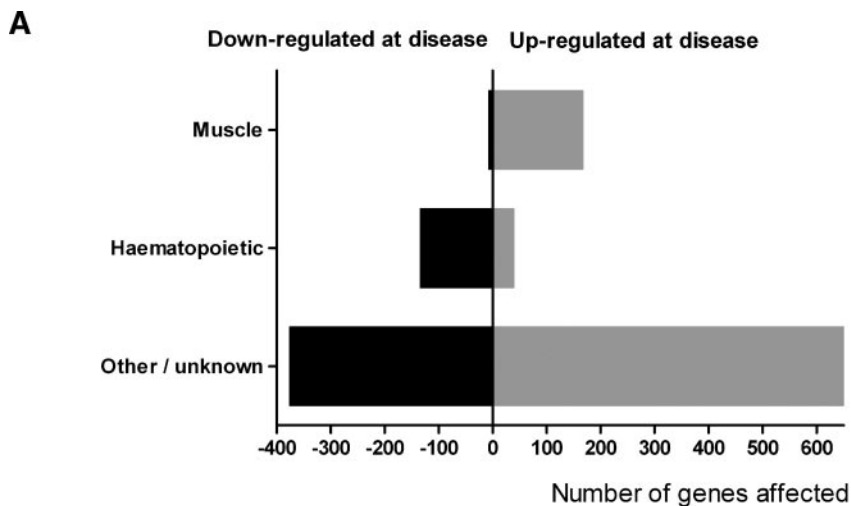
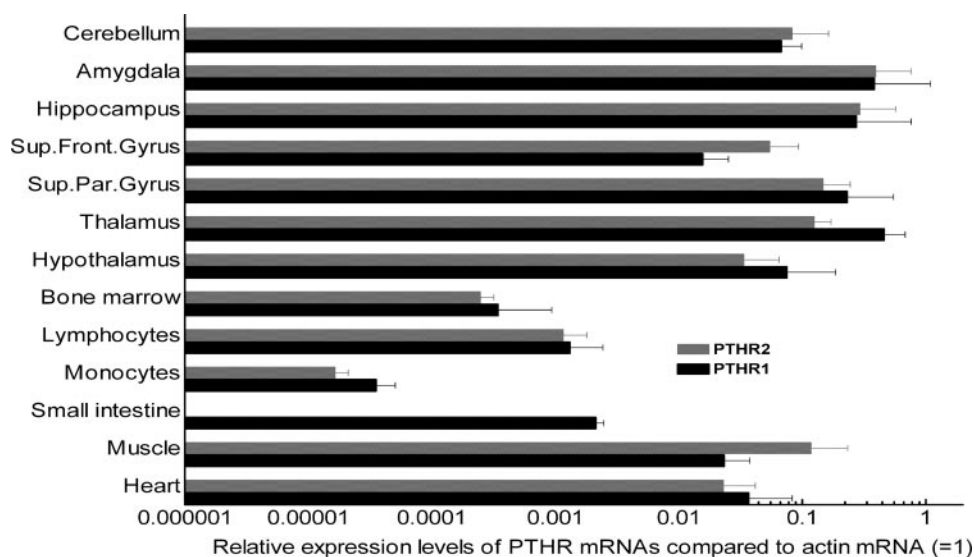


Fig. 1. A: overview of tissue-related genes differentially expressed pre- and postoperatively in primary hyperparathyroidism (PHPT) and fulfilling statistical *criterion 1* or *2* for filtering. Most muscle tissue-related mRNAs are increased with disease, in contrast to hematopoietic tissue mRNAs. B: individual expression of mRNAs characteristic of muscle (119) (*a*) and hematopoietic cells (88) (*b*) in each of 7 patients, representing the most significantly regulated genes (1,388). Each gene is displayed as a bar and expressed as a ratio in log₂ scale between pre- and post-operative mRNA values for each individual patient. Statistical *criterion 1* and *2* for filtering were fulfilled (see METHODS).

Fig. 2. Expression levels of PTH receptors 1 and 2 (PTH1R and PTH2R) mRNAs in various tissues relative to actin mRNA analyzed by real-time RT-PCR. mRNA levels were tested in triplicate from 3 different persons. Bars illustrate means (SD).



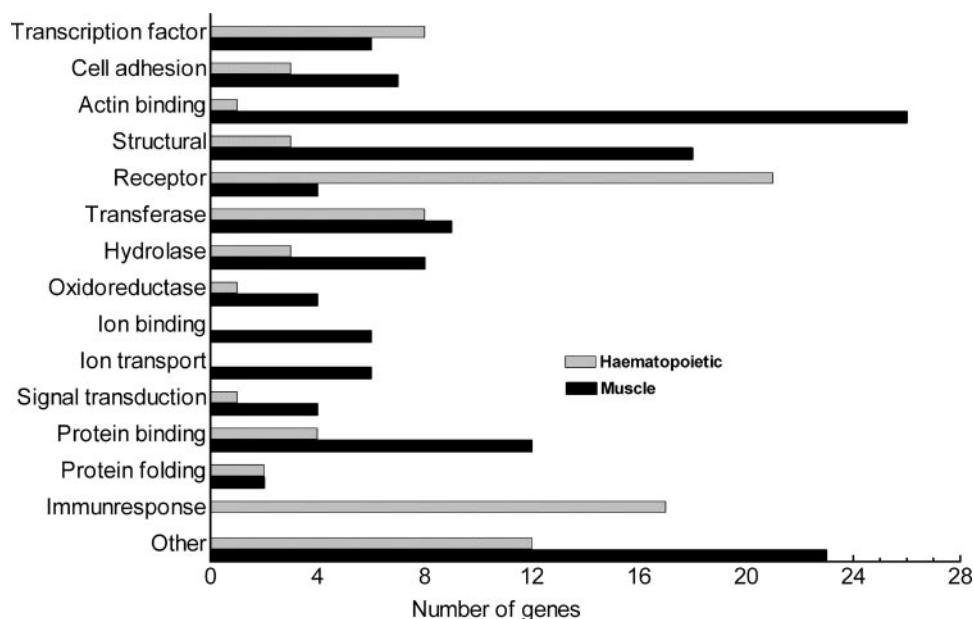
and PTH2R mRNAs were both present in all human tissues and cells tested, except for the small intestine, where only PTH1R mRNA was detected (Fig. 2). The abundant and uniform representation of the two receptor mRNAs in the CNS, especially in the cerebellum, amygdala, hippocampus, superior frontal gyrus, superior parietal gyrus, thalamus, and hypothalamus is noteworthy, reaching almost the level of actin (Fig. 2). Cardiac and skeletal muscle contain, on average, about one-tenth of the mRNA amounts present in the most enriched regions of the brain. Thus, the prerequisite for PTH to exert a direct action on muscle and hematopoietic tissue/cells is present.

Differential expression of muscle- and hematopoiesis-related mRNAs in PHPT. Functional groups of genes were identified using annotations in the NetAffx analysis center and from Eli Lilly as a second validation. The results were highly comparable. The number of differentially displayed genes in each functional category is presented in Fig. 3. Among muscle-

related transcripts there was an overrepresentation in the groups "actin binding" and "protein binding." Also, mRNAs coding for ion binding and transport proteins were prominent. Alterations in immune response and immune receptor genes were the most striking features among the hematopoiesis-related genes. The number of affected mRNAs for transcription factors and transferases seemed to be similar in hematopoiesis and muscle-characteristic genes (Fig. 3). The mRNAs defining muscle proteins were related especially to energy processes (e.g., creatine transferases) and to proteins participating in cellular contractility and ion binding and transport (Figs. 3 and 4).

Individual changes in each patient describing 50 of the statistically most affected genes in each functional category characteristic of the two tissues are depicted in Fig. 4. By compiling the data, reproducibility and consistency of the gene expression results during disease in *patients 1–6* were visualized. During disease it was characteristic that mRNAs related to the contractile and energy providing systems were most

Fig. 3. Functional classification of the most significantly regulated genes between pre- and postoperative values for mRNAs. For statistical criteria, see Fig. 1.



increased in muscle (Fig. 4A). Also, mRNAs for ion binding/transport and transcription factors were highly regulated in the patient biopsies before compared with after PTX. Some muscle-related mRNAs were increased up to 10-fold (e.g., myozenin) during PHPT, whereas the majority were two- to fourfold increased (Fig. 4A) (see <http://www.med.uio.no/imb/medbiokj/kgautvik/index.html> for the genes affected in this and the other affected canonical pathways). Of the 50 muscle-related mRNAs depicted in Fig. 4A, only plakophilin-4 was reduced (almost 3-fold), whereas several other muscle-related mRNAs also were significantly reduced in PHPT (chondroitin sulfate, GalNAcT-2, ganglioside-induced differentiation-associated protein-1, syntrophin, β 1, glycerol-3-phosphate dehydrogenase-2, calcitonin receptor-like; Fig. 1B, a, but not shown in Fig. 4A). These do not represent members of any obvious particular group or pathway, and their clinical translation appears unclear.

During PHPT, some mRNAs related to hematopoietic functions were increased, e.g., hepatic leukemia factor (HLF), chemokines, and some mRNAs related to DNA binding and transcription factors (Fig. 4B). The median degree of hematopoietic mRNA alteration (up/down) was $\sim 45\%$, whereas most of the downregulated mRNAs were in fact reduced by $\sim 75\%$, e.g., immunoglobulin heavy constant- γ (IGHG1; Fig. 4B). In addition, receptor-signal transduction, certain chemokines, e.g., IL-4, and transcription factors represented major mRNA groups that were highly affected before and after curative surgery.

To validate the findings of transcript measurements by the microarray approach, we repeated the measurements of selected mRNA levels using real-time RT-PCR on RNA from patients 1, 2, 4, 5, and 6 (patient 3 was omitted due to limited amounts of RNA). The results demonstrated a very good consistency with the findings obtained using microchip analysis and in general showed an even more pronounced change in expression (Fig. 4).

The data have been submitted to the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository (acc. no. E-MEXP-847).

Identification of affected canonical pathways at PHPT and clinical significance. A total of 1,388 genes that fulfilled statistical criterion 1 or 2 (see Fig. 1A) (METHODS) were analyzed by canonical IPA (Ingenuity Systems, www.ingenuity.com).

The canonical pathways present in the IPA library and showing highest significance for our data set are displayed in Table 1.

Several pathways that were not assigned to a predominant tissue, but are of general biological importance, included energy metabolism represented by oxidative phosphorylation, citrate cycle, pyruvate metabolism, pentose phosphate pathway, and ubiquinone synthesis, all of which were strongly and highly significantly affected during PHPT, showing an overall increased expression pattern. It is of particular interest that the "calcium-signaling" pathway, known to be essential in muscle and blood cell functions, is by far the most affected system, in which 50 of 172 mRNAs ($\sim 30\%$) are altered. In agreement with the profound alterations in the mRNA profiles of cell receptors, cell signaling and chemokines as related to both muscle and hematopoietic tissue/cells (Fig. 4) and the molecular networks reflecting calcium and β -adrenergic signal transductions were statistically most, and quite remarkably, pre-

dominantly increased during disease. Calcium plays essential roles in skeletal and cardiac signal-contraction coupling but is also of central importance for several hematological/immunological cellular functions. For instance, we find that several mRNAs within the calcium-signaling pathway (CRAC, MEF2, DSCR1, RyR) are affected in PHPT. Their corresponding proteins participate in immune cell activation as well as in B and T cell development via activation of the transcription factor NFATC (the pathway and affected genes are illustrated at <http://www.med.uio.no/imb/medbiokj/kgautvik/canonical.html> and reviewed in Ref. 9). The 11 genes affected and present in the β -adrenergic signaling respond to the catecholamines nor-epinephrine and epinephrine, providing coordinated control of contractility, metabolism, and gene regulation, and can be viewed at <http://www.med.uio.no/imb/medbiokj/kgautvik/canonical.html> (reviewed in Ref. 37). It is well known that an upset regulation of the β -adrenergic system, which appears also to be greatly overactivated in patients with PHPT, will cause erroneous cardiac muscle cell function and, if of longer duration, may lead to heart symptoms (reviewed in Ref. 25).

Other regulated pathways with long-term potential clinical consequences are phenylalanine, tyrosine, and tryptophan biosynthesis and Parkinson's signaling [a signaling cascade that in brain leads to the death of dopaminergic neurons (reviewed in Ref. 21)]. It is noteworthy that seven of nine enzyme complexes in the oxidative phosphorylation chain were among the most significantly altered, causing a potentially important imbalance of muscle cell energy requirement (Table 1 and website <http://www.med.uio.no/imb/medbiokj/kgautvik/canonical.html>).

DISCUSSION

The results show that patients with asymptomatic, mild PHPT without known clinical organ involvement have a marked, disease-dependent change in expression of mRNAs characteristic of muscle- and hematopoiesis-related genes as previously described for bone related genes (35). This dysregulation of a large number of genes related to muscle and hematopoiesis during PHPT has not previously been recognized. Because the pathological biochemical parameters and loss of bone mineral density were reversible after the operation, it is reasonable to attribute the molecular changes to being caused by the disease. Also, changes in several bone-associated mRNAs (e.g., osteopontin, osteocalcin, fibronectin-1, and several collagens, including 1A1 and 1A2) previously shown to be affected by PTH overstimulation (35) may be argued to represent a validation of the results even if the number of patients is limited. All of the patients had high serum PTH while showing small or modest rises in serum Ca^{2+} . Since the median changes in serum PTH and Ca^{2+} during disease were 5- and 1.25-fold, respectively, it is tempting to regard PTH, and not serum Ca^{2+} , as a major cause of the molecular derangement. In support of this view, it may be argued that, since the local $[\text{Ca}^{2+}]$ in bone marrow adjacent to trabeculi can be very high irrespective of disease (40), a mere 25% increase in $[\text{Ca}^{2+}]$ would hardly have a significant effect on the hematopoietic stem cells that are closely associated with bone. Furthermore, the fourth Tromsø Epidemiological Study, involving nearly 7,000 persons, demonstrated a clear relationship between left ventricular hypertrophy and serum PTH with no relation to serum calcium levels (36). As serum 1,25(OH) $_2$ D is

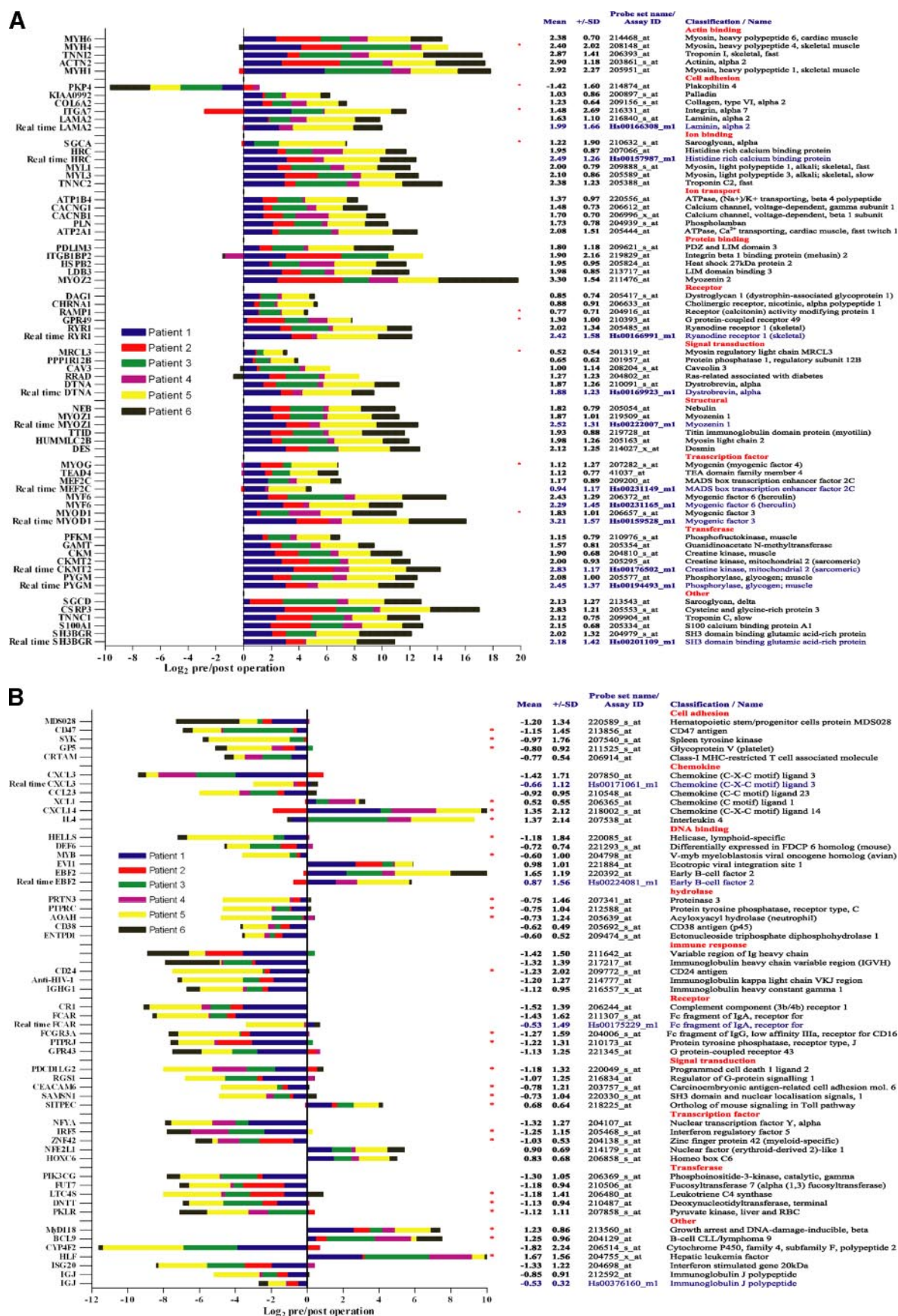


Table 1. *Canonical pathways affected in PHPT*

Pathway	Ratio Affected/Total	Number Up/Downregulated in PHPT	Significance (Fischer's Test)
Calcium signaling	50/112	46/4	0.000
Cardiac β -adrenergic signaling	19/77	13/6	0.000
Oxidative phosphorylation	28/143	27/1	0.001
Citrate cycle	8/27	7/1	0.007
Phenylalanine, tyrosine, and tryptophan biosynthesis	5/14	3/2	0.012
Pyruvate metabolism	14/70	12/2	0.016
Pentose phosphate pathway	8/33	5/3	0.028
Parkinson's signaling	5/17	2/3	0.029
Ubiquinone biosynthesis	10/49	10/-	0.036

Pathways within the Ingenuity Pathways Knowledge Base that were most significantly regulated among the 1,388 genes fulfilling statistical *criterion 1* or 2 (see METHODS). PHPT, primary hyperparathyroidism. Significance of the association between the data set and the canonical pathway were analyzed in 2 ways: 1) ratio of no. of genes from the data set that map to the pathway divided by the total no. of genes that map to the canonical; 2) Fischer's exact test, used to calculate a *P* value testing the hypothesis that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

also increased in PHPT (35), a contributing effect on gene expression is possible due to increased activation of the vitamin D₃ receptors, since they are ubiquitously expressed (8, 12). Vitamin D has antiproliferative effects on hematopoietic cells mediated via induction of the cyclin-dependent kinase (CDK) inhibitors p27^{kip1} and p21^{WAF1/CIP1} as well as via enhanced TGF- β signaling (reviewed in Refs. 28 and 44). The CDK inhibitor p27^{kip1} mRNA is present but not changed, whereas p21^{WAF1/CIP1} mRNA appears to be slightly increased. Although mRNAs encoding the TGF- β 1 and SMAD's mediating TGF- β signaling are not changed in PHPT, the TGF- β RI and TGF- β RII mRNAs are slightly decreased [Array Express Repository (E-MEXP-847)]. To what degree altered vitamin D level is responsible for these small changes and what effects they may have on expression of muscle- and hematopoiesis-associated genes in PHPT is unclear. The presence of several different cell types within the biopsies and tissues makes the assignment and translation of molecular signals to cell function difficult.

Except for bone and kidney, chronic receptor activation by PTH in PHPT has not earlier been strongly linked to particular clinical symptoms, possibly because CNS- and muscle-related complaints have been difficult to assess objectively in medical investigations. Morphological changes have been described in cases where myopathy was part of the clinical picture in PHPT (6), and it is notable that such changes have not been described in other hypercalcemic conditions.

Recent knowledge indicates that several tissues express many of the same type of genes. For example, isolated osteocytes, which have been shown to be PTH dependent (30), express many genes characteristic of muscles and CNS (18).

The present study therefore indicates that chronic PTH stimulation affects defined groups of mRNAs expressed in all target cells. It therefore became important to better define the human target cells/tissues for PTH in order to understand the observed molecular changes in relation to clinical cell and organ pathology. Muscle function is heavily dependent on a continuous energy supply, and the increased expression of genes related to energy and metabolism will probably also lead to constant and inappropriately elevated ATP generation when not linked to muscular activity. Therefore, muscle cells in PHPT may develop a state of energy insufficiency's being relevant in explaining muscular atrophy, weakness, and fatigue, which are commonly reported by patients with this disease (29). The canonical pathway most significantly affected was calcium signaling, in which 50 of 172 mRNAs showed changed expression during disease. Most of these mRNAs encode proteins participating in the typical muscle contractile process (myosins, troponins, tropomyosins, Ca²⁺-binding proteins, and Ca²⁺ channel proteins). It is also a striking feature of the discovered molecular pathology that it probably also involves the cardiac β -adrenergic system present in cardiomyocytes. This finding may help explain the dysregulation of cardiac function and development of left ventricular hypertrophy and failure representing the main cause of premature death in patients with PHPT (20, 36).

Also, expression of genes that regulate proliferation and differentiation of muscle-related cells was altered in patients with PHPT: MyoD1, which induces the transcription factors myogenin (increased in PHPT) and MRF4 (reviewed in Ref. 5), and calpain 3. The latter is a protease participating in sarcomere remodeling by cleaving filamin C involved in myocyte differentiation (50). A possible correlation between the degree of clinical severity of muscle and CNS affection, and extent of molecular derangement, must await further studies, since the present patients had early disease and did not present typical clinical morbidity or biochemical blood changes; e.g., serum creatinine levels were normal (data not shown).

The association between PHPT and immunological dysfunction is not recognized today but may be of clinical importance. Reduced resistance to infections and increased prevalence and premature death of malignant diseases in PHPT have been reported (1, 3, 11, 13, 20, 32). A relationship between PHPT and infection is described as being the fourth most prevalent cause of premature death (20). Also, the high prevalence of *Helicobacter pylori* in patients with PHPT (11) and urinary tract infections in 26% of 201 tested dogs with PHPT (13) points to an increased susceptibility to infections. Of the 55 different genes described by Affymetrix to affect the innate immune system, three showed significantly reduced expression in PHPT [complement component 7, complement component (3b/4b) receptor 1, and toll-like receptor-1], whereas one (clusterin) was increased. In our patients with PHPT, a number of chemokine mRNAs were altered and, e.g., IL-4 mRNA was increased ~2.6-fold (log₂ = 1.37; Fig. 4B). It is of interest that

Fig. 4. Cumulative changes of regulated known mRNAs shown as pre- and postoperative ratios and grouped into functional categories. In both panels, a selection of the 5 most regulated mRNAs in 10 different groups is displayed. Each bar shows the compiled change in 1 gene for each patient. Results from real-time RT-PCR are shown as an adjacent bar. Means (SD) of patients 1, 2, 4, 5, and 6 are listed for real-time RT-PCR results, while patient 3 is also included in results from microchip analysis. A: muscle. B: hematopoietic cells. Genes labeled with an asterisk fulfill *criterion 1* or 2 for filtering and statistical analysis on parathyroidectomy (PTX), whereas those not marked fulfill both criteria. Tables with all muscle- and hematopoiesis-regulated mRNAs are displayed at <http://www.med.uio.no/imb/medbiokj/kgautvik/canonical.html>.

production of IL-6, sIL-6R, and TNF- α from cultured white blood cells is elevated in patients with PHPT before operation, showing a PTH-dependent biochemical abnormality in a hematological cell lineage (15–17, 34) (IL-4 was not analyzed in blood). These molecular and biochemical pathways point to a possible clinical relationship. In this respect, it is of interest that PTH(1–34) treatment of mice caused expansion of hematopoietic stem cell precursors (4). Furthermore, we find mRNAs for immunoglobulins, immunoglobulin receptors, signal transduction mediators, and growth factors to be altered in PHPT. A possible reduced response to antigens may contribute to the increased susceptibility to cancer and infections.

Mesenchymal cells in the bone marrow are the source of osteogenic cell differentiation and osteoblastic cells, which together with immune cell precursors constitute the hematopoietic stem cell niche (4). The Wnt pathway is central in both osteoblast and hematopoietic differentiation and proliferation. Interestingly, several genes involved in Wnt signaling show significantly higher expression in PHPT [Wnt5A, Wnt3, frizzled 7, dishevelled 1, LRP16, and Dickkopf (Dkk) 2; Array Express Repository (E-MEXP-847)], suggesting a role for some of these factors in contributing to the pathological picture in PHPT. mRNAs encoding downstream mediators of the Wnt pathway (β -catenin, GSK-3 β , LEF-1/T cell factor) were not significantly altered or were undetectable on the chip.

Because PTHR1, but not the PTHR2, was found to be significantly (2-fold) increased in disease, thereby providing a molecular basis for PTH target cell dysregulation, we examined in some detail their distribution in human CNS, muscle, and blood cells. By real-time RT-PCR analysis we showed the presence of both PTHR1 and PTHR2 mRNAs in human monocytes, lymphocytes, bone marrow, and heart muscle, and both receptor mRNAs were markedly expressed in several regions of the brain. These results, which add to and extend previous knowledge (39), offer a molecular understanding to explain the CNS and muscle pathophysiology in PHPT.

In gene-modulated mice, bone-specific expression of constitutively active PTHR1 led to perturbed hematopoiesis and a greatly reduced number of clonogenic stromal cells (27). Furthermore, other animal studies have shown that osteopontin reduces the hematopoietic stem cell pool (45). In PHPT we have observed activation of PTH1R as well as increased osteopontin mRNA levels, as shown previously (35). Thus, one interesting hypothesis warranting further research is that the number of hematopoietic stem cells may be reduced in PHPT as a consequence of chronic PTH stimulation, an effect opposite to the action of brief PTH infusion (4). The profound changes observed in specific molecular patterns analyzed by global gene expression as demonstrated in our study suggest a number of mechanisms that may translate to and well explain recognized clinical consequences of PHPT, providing strong directions for further research. The present results indicate that early diagnosis and treatment of these patients may avoid irreversible molecular pathology.

ACKNOWLEDGMENTS

Assistance from Ståle Nygård regarding statistical analyses is gratefully acknowledged.

GRANTS

S. Reppe was financed by the Norwegian Foundation for Health and Rehabilitation through the Norwegian Women's Health Association. We are

also grateful for previous support from the Norwegian Osteoporosis Society, the Norwegian Research Council, Anders Jahre's Foundation for Promotion of Science, Rachel and Otto Bruuns Legate, and the Novo Nordisk Foundation. This work is part of the European Union project OSTEOGENE (no. FP6-502491), which also receives financial support from the Norwegian Health Region East and Ullevaal University Hospital.

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